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Hepatic Effects of a Phthalate Ester Plasticizer Leached from Poly(vinyl Chloride) Blood Bags Following Transfusion

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The effects of di(2-ethylhexyl) phthalate (DEHP) on hepatic function and histology were evaluated in the rhesus monkey undergoing platelet and plasma transfusion. The average cumulative amount infused in one year is comparable to that received by patients who undergo chronic transfusion. Abnormalities in hepatic scan and BSP kinetics persisted for up to 26 months after transfusion, as did histologic abnormalities. Patients undergoing maintenance hemodialysis receive a yearly dose of DEHP which is 10-20 times that which produced hepatotoxicity in the transfused rhesus.

The plasticizer di(2-ethylhexyl) phthalate (DEHP) is incorporated into poly(vinyl chloride) (PVC) medical devices to produce flexibility. PVC medical devices such as blood bags and indwelling intravenous catheters contain up to 40% by weight of DEHP. This material is leached from PVC by blood and lipoprotein-containing solutions (*1*). This solubilization is time- and temperature-dependent (*1*). Previous studies have demonstrated DEHP in the tissues of multitransfused patients and in neonates whose only exposure was indwelling umbilical catheters (*2*, *3*).

Jacobson and Kevy (*4*) have demonstrated that DEHP levels of 5 mg/dl, the concentration found in whole blood stored for 21 days at 4°C and in platelets stored at 22°C for 24 hr, significantly inhibits the growth of human diploid fibroblasts. As a consequence of this finding, rhesus monkeys were used in a chronic transfusion study in which PVC blood bags were compared with those made of polyethylene.

The rhesus monkey was chosen as the animal model, since there is marked species difference in the metabolism of DEHP. Their baseline serum protein analysis and agarose electrophoresis pattern are normal in terms of human values except for the orosomucoid level which is slightly lower. In addition they behave similarly to both children and adults as far as compartmentalization analysis of bromosulfophthalein (BSP) kinetics and hepatic ^{99m}Technetium-labeled sulfur colloid scanning techniques.

These animals were transfused with either platelet-rich or platelet-poor plasma in a manner identical to that utilized in clinical medicine. This is an extremely critical point, because virtually all the toxicology literature relative to DEHP is based upon rodent exposure in a manner which in no way mimics the way the human is exposed via transfusion of blood products or renal dialysis.

Materials and Methods

Experimental Groups

Immature rhesus monkeys approximately six months old and weighing less than 5 lb were divided into the four following groups.

PVC Platelet-Rich Plasma (PRP) Transfused. Three rhesus monkeys were transfused weekly for one year with 15 ml of PRP stored for 48 hr at 22°C, and two monkeys received PRP stored at 4°C.

PVC Platelet-Poor Plasma (Plasma) Transfused. Two monkeys were transfused biweekly for 6

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months with 15 ml of plasma stored at 22°C for 5 days. Even though this is no longer standard blood bank practice, plasma was stored at room temperature to maximize the leaching of DEHP.

Nontransfused Controls. Three monkeys lived in the same environment and underwent the same test procedures as the experimental rhesus.

Transfused Controls. Two monkeys were transfused weekly for 6 months with 15 ml of PRP stored for 48 hr at 22°C in polyethylene blood bags.

An aliquot of each transfusion was quantitated for DEHP content.

Preparation of Platelet-Rich Plasma

Adult donor monkeys were plasmapheresed biweekly to obtain platelet-rich plasma. Blood, 100 ml, was collected into a 150 ml PVC or polyethylene blood pack aseptically filled with 16 ml ACD anticoagulant. The blood was centrifuged in a SBV No. 1 International centrifuge at 500 *g* at room temperature for 10 min, and the platelet-rich plasma was aseptically transferred into a second 150 ml PVC or polyethylene transfer pack. These packs were then stored at 4 or 22°C for 48 hr. The average platelet count in each bag was 150,000/mm^3. The red cells were returned to the donor monkeys.

Preparation of Plasma

Adult donor monkeys were plasmapheresed into PVC blood packs as detailed above, except that the whole blood was centrifuged at 2520 *g* for 10 min in a RC-3 Sorvall centrifuge. The plasma was transferred into a PVC transfer pack and stored at 22°C for up to 5 days. Each unit of plasma was tested and found to be platelet free.

DEHP Quantitation

Levels of DEHP in blood products, blood and tissue were determined by gas chromatography with the use of our modification of the method of Jaeger (*4*). The sensitivity of the assay is 0.1 ppm (parts per million).

DEHP Dosage

The amount of DEHP received by an experimental animal was calculated as the number of milliliters of stored blood component received times its DEHP concentration. The total amount of DEHP was determined by a summation of the results of each transfusion.

Hepatic Scintillation Scanning Techniques

^{99m}Technetium-labeled sulfur colloid provides stable count rates, is short-lived, and has favorable radiation dosimetry (*5*). Each rhesus monkey received ^{99m}Tc at dose of 0.015 mCi/kg body weight. Within 45 min after injection, the abdomen was scanned. Peak counts were recorded over the substance of the liver and spleen for 3 min and repeated five times. The hepatosplenic ratio was then determined from the mean counts over the liver and the spleen.

Sulfobromophthalein (BSP) Transport

Sulfobromophthalein (BSP) transport was evaluated by the method of Quarfordt and associates (*6*). Each study was performed after at least a 12-hr fast. BSP was injected intravenously in a dose of 5 mg/kg of body weight. Sequential blood samples were obtained from the arm opposite that used for injection. Zero time for the study was set at the midpoint of the injection, which took an average of 30 sec to complete. Blood samples were obtained prior to injection for BSP recovery studies, then at 3-min intervals for 30 min, and thereafter at 15-min intervals for up to 2 hr. After the final blood sample, urine was collected for BSP recovery.

The plasma disappearance curve following a single injection of BSP was analyzed by a computer technique for determining compartmental flow. The method computes the hepatic removal rate (HRR), hepatic to plasma reflux rate (H-PRR), the biliary secretory rate (BSR), and the urinary excretion rate (UER) by means of the simulation analysis and modeling technique of Berman and associates, with the use of the SAAM digital computer program on an IBM 370-5165 computer (*7*). These rates are expressed as the fractional rate (i.e., percent transported) per minute.

The model was verified in the rhesus monkey by measurement of the disappearance of dye from the plasma and quantitation of the concentration of dye excreted in the bile and urine. The bile samples were obtained by cannulation of the common bile duct. The experimentally observed values were plotted along with the computer-calculated values and the curves were superimposed. An injected dose was established which would not saturate the transport processes. Data obtained by these methods were compared to previously reported studies by us (*8*) in normal children and adults and children with cystic fibrosis (CF).

Histopathology

Multiple surgical liver biopsies were performed on all transfused and nontransfused monkeys. In order to ensure adequate sampling for all studies, a wedge biopsy was obtained on each occasion. Tissues were rapidly fixed in formalin and embedded in paraffin. Sections were stained by hematoxylin-eosin, periodic acid-Schiff, periodic acid-Schiff-alcohol and Sudan Black. All preparations were examined in blinded fashion by four observers.

Results

DEHP Dosage

At the start of this study ten consecutive patients were identified who predictably would be multi-transfused. During the course of one year, each unit of red cells, whole blood, plasma and platelets was quantitated for DEHP. The amount of DEHP the patients received ranged from 56 to 1500 mg or 2.1 to 27.5 mg/kg. During their one-year period of transfusion the group infused with platelet-rich plasma stored at 22°C received 76.8-96.0 mg of DEHP or 24-30 mg/kg, those transfused with platelet-rich plasma stored at 4°C received 20.5–27.0 mg or 6.6-8.7 mg/kg and those infused with plasma 108 mg or 33 mg/kg.

Assessment of Liver Function

^{99m}Tc Sulfur Colloid Liver-Spleen Scans. Severe or moderate impairment of hepatic perfusion and/or infiltration are detected by irregular distribution of tracer within the liver. Milder forms of parenchymal disease can be quantitated by determination of the distribution ratio of radiocolloid between liver and spleen. Each animal served as its own control. All subsequent determinations of the hepatosplenic ratio were compared to the baseline ratio obtained prior to transfusion. Liver/spleen activity ratios were calculated assuming the spleen to be one. A ratio was considered abnormal if there was at least a 30% decrease from the baseline level.

The results of the scanning studies in all monkeys are shown in Table 1. All rhesus transfused with platelets stored in PVC had a significant decrease in ratio throughout the period of transfusion therapy. One rhesus in each of the platelet transfusion groups were sacrificed at varying intervals during this study to determine the DEHP organ content. Three of the five surviving monkeys who were transfused from PVC-DEHP blood bags demonstrated abnormal scans for up to the 26 months post-transfusion that they were followed during the course of this study.

There were no changes in the hepatosplenic ratios in the monkeys transfused with platelets stored in polyethylene or in the nontransfused controls.

BSP Transport. The plasma disappearance curves for BSP and the data from the two-compartment model of Quarfordt provide a very sensitive indicator of subclinical liver disease. We have demonstrated that the transport of BSP in the normal monkey and the normal human being are identical. The normal immature rhesus monkey and the normal child have a single exponential curve, whereas the normal adult rhesus monkey and the normal adult human have a biexponential curve.

The change in the plasma BSP disappearance curve is proportional to the degree of liver involvement. A child with cyctic fibrosis (CF) who has no evidence of hepatomegaly and normal liver function demonstrates a single exponential disappearance curve identical to that of a normal child. The child with CF who has hepatomegaly and biopsy documented liver pathology demonstrates a biexponential disappearance curve, even though SGOT, SGPT, alkaline phosphatase, bilirubin and standard BSP tests are normal. The development of a biexponential disappearance curve in a child or immature subhuman primate, therefore, serves as a sensitive marker of hepatic dysfunction and is considered abnormal. One can also distinguish in the adult the difference between a normal biexponential disappearance curve and that produced by liver disease (*8*).

Table 1. Rhesus liver/spleen activity ratios following ^{99m} Tc sulfur colloid infusion.

	Abnormal/normal					
		Transfusion interval		Interval after transfusion		
Rhesus group	Baseline	6 mo.	12 mo.	5 mo.	14 mo.	26 mo.
Nontransfused	0/3	0/3	0/3	0/3	0/3	0/3
22°C – PRP – PVC	0/3	3/3	3/3	2/3	2/3	1/2
4°C – PRP – PVC	0/2	2/2	2/2	1/1	1/1	1/1
Plasma – PVC	0/2	1/2	–	1/2	1/2	1/2
PRP – Polyethylene	0/2	0/2	0/2	0/2	0/2	0/2

The results of the compartmental analysis of BSP kinetics in the transfused and nontransfused immature rhesus monkeys are shown in Table 2. A BSP analysis was considered abnormal if the disappearance curve was biexponential. The monkeys transfused with platelets stored in polyethylene and the nontransfused controls did not exhibit any abnormalities throughout the course of this study. Four of the five monkeys transfused with platelets stored in PVC exhibited abnormal disappearance curves following one year of platelet transfusions. This persisted in three of the four remaining rhesus monkeys 14 months after transfusion and in one rhesus 26 months following cessation of transfusions. Both rhesus transfused with plasma stored in PVC had abnormal BSP disappearance curves immediately following the six months of transfusion. These reverted to normal within five months following the cessation of transfusions.

Liver Histopathology. A biopsy was considered abnormal when at least two of the following findings were present: (1) foci of parenchymal necrosis (aggregates of chronic inflammatory cells and Kupffer cells in the parenchymal with degenerating hepatocytes); (2) vacuolization of Kupffer cells; (3) chronic inflammatory cell infiltrates; and (4) prominence and/or increase in the number of Kupffer cells and the presence of binucleate cells. At no time during this study were any histologic abnormalities identified in liver biopsies of nontransfused or polyethylene-transfused control monkeys.

All of the animals transfused with platelets or plasma stored in PVC demonstrated histologic abnormalities throughout the follow-up period.

Figure 1 demonstrates finding obtained in biopsy 24 months following cessation of transfusion in a monkey who had received platelets stored at 4°C. Figure 2 demonstrates the persisting histologic findings obtained at necropsy 32 months after the cessation of transfusions in one of the monkeys transfused with platelet-rich plasma stored at 22°C.

Histologic changes at this time were characterized by aggregates of inflammatory cells, hepatocyte degeneration, and multinucleated and binucleated giant cells. These findings persisted despite the fact that no DEHP was detected in liver tissue at the time of necropsy.

Tissue Levels of DEHP

One of the inherent difficulties of liver biopsies is that only small specimens can be obtained. Tissue samples obtained at biopsy averaged 225 mg wet weight (range 100–400 mg). The limit of sensitivity of our analysis for DEHP by G.C. is 0.1 ppm. Any samples with DEHP contents of 1.4 ppm to 1.5 ppm, levels well above background, were considered to have only a trace amount. Table 3 lists the DEHP content of liver tissue obtained both at biopsy and necropsy.

As shown in Table 3, the initial biopsies of all the PVC transfused rhesus contained significant amounts of DEHP. The nontransfused and polyethylene-transfused monkeys had no detectable DEHP. The five month follow-up samples contained concentrations similar to those initially measured with the exception of one PRP-transfused monkey and the two plasma-transfused animals that only had trace amounts of DEHP. At the 14 month follow-up biopsy of the PRP animals, three had only trace amounts of DEHP, and one had no detectable level. Since these samples were in the lower range of the sensitivity of our assay for DEHP, we sacrificed one of the animals (rhesus "G"). The tissue samples utilized ranged from 2.5 to 4.0 g wet weight. Significant levels of DEHP were found in the liver, testis, heart and omental fat. The total residual organ level, excluding fat, was less than one percent of the dose administered.

DEHP was not detected in liver samples obtained at necropsy 32 months after transfusion, despite the persistence of abnormal histologic findings. Unfortunately, none of the samples were analyzed for the monoester (MEHP).

Hemodialysis Studies

Since the rate of leaching of DEHP from PVC is dependent on the temperature and lipid content of

Table 2. Rhesus BSP kinetics.

	Abnormal/normal					
		Transfusion interval		Interval after transfusion		
Rhesus group	Baseline	6 mo.	12 mo.	5 mo.	14 mo.	26 mo.
Nontransfused	0/3	0/3	0/3	0/3	0/3	0/3
22°C - PRP - PVC	0/3	3/3	3/3	2/3	2/3	1/2
4°C - PRP - PVC	0/2	0/2	1/2	1/1	1/1	0/1
Plasma - PVC	0/2	2/2	–	0/2	0/2	0/2
PRP - Polyethylene	0/2	0/2	0/2	0/2	0/2	0/2

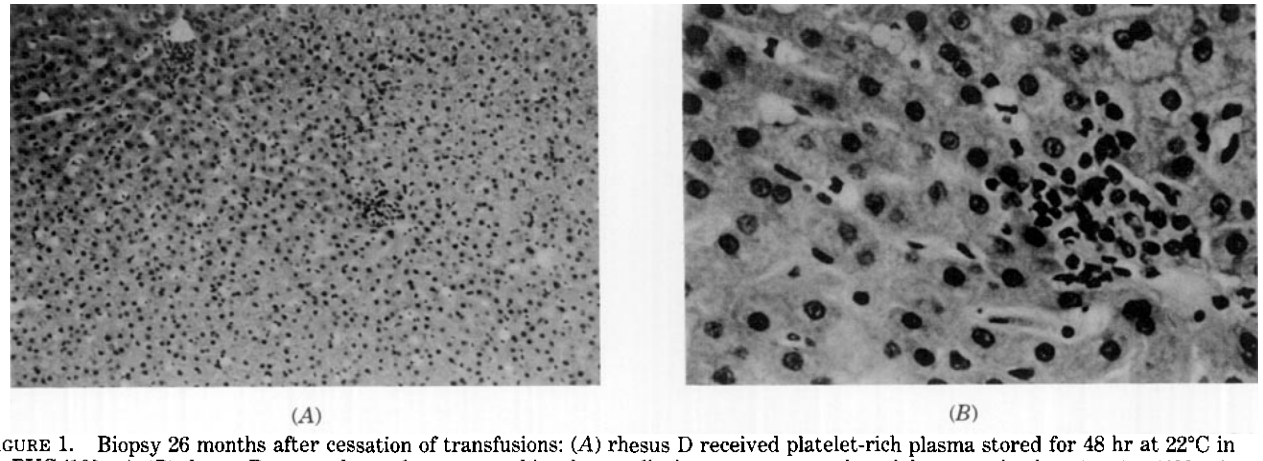

FIGURE 1. Biopsy 26 months after cessation of transfusions: (*A*) rhesus D received platelet-rich plasma stored for 48 hr at 22°C in PVC (100 ×); (*B*) rhesus D; areas shown demonstrate binucleate cells, hepatocyte necrosis and degenerating hepatocytes (400 ×).

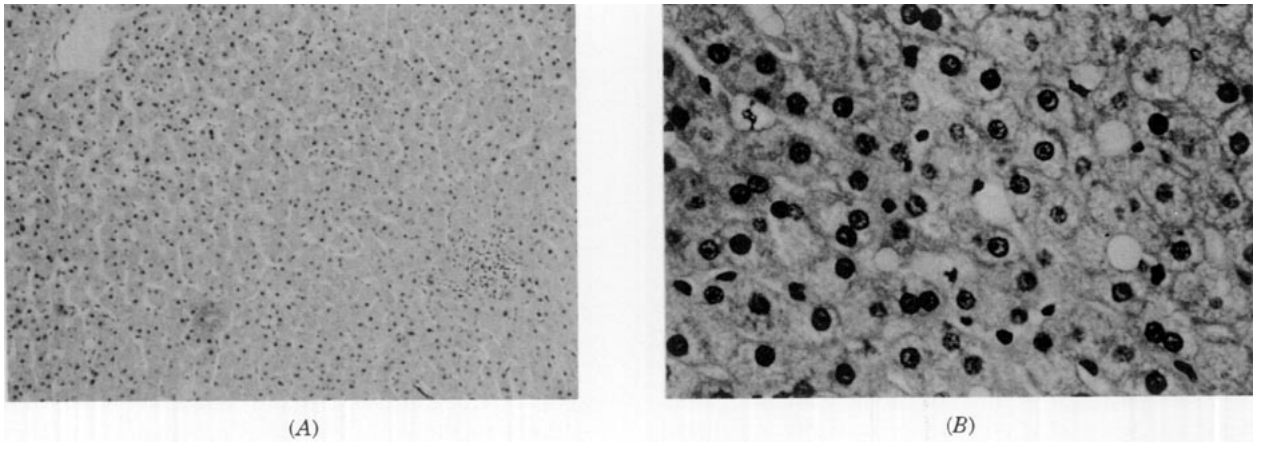

FIGURE 2. Necropsy 32 months after cessation of transfusions: (*A*) 100×; (*B*) 400×. Areas shown demonstrate aggregates of inflammatory cells, hepatocyte necrosis and binucleate cells. DEHP content: none detected.

Table 3. DEHP content in liver of biopsied monkeys.[a]

Animals	Procedure	Total DEHP infused, mg	DEHP content, μg/g wet weight of liver				
			Initial biopsy	Interval after transfusion			
				5 mo.	14 mo.	26 mo.	32 mo. (necropsy)
B	NT[b]	0	ND[c]	ND	ND	ND	ND
C	NT	0	ND	ND	ND	ND	ND
M	NT	0	ND	ND	ND	ND	ND
D	22°C (PVC)–PRP	74.80	3.0	Trace	ND	6.7	ND
F	22°C (PVC)–PRP	96.00	5.0	3.8	Trace	ND	ND
G	22°C (PVC)–PRP	78.72	10.0	9.2	0.7 (Necropsy)	Deceased	
E	4°C (PVC)–PRP	27.00	1.0	1.8	Deceased		
H	4°C (PVC)–PRP	20.52	1.5	3.1	Trace	Deceased	
S	22°C (PVC)Plasma	96.00	10.1	Trace	Trace	ND (Necropsy)	
T	22°C (PVC)Plasma	108.00	18.1	Trace	18.7	Trace (Necropsy)	
L	22°C (PE)–PRP	0	ND	ND	ND	ND	
P	22°C (PE)–PRP	0	ND	ND	ND	ND	

[a]Initial biopsy performed on PRP animals after 1 year of duration; initial biopsy performed on plasma animals after 6 months of transfusion.

[b]NT = not transfused.

[c]ND = none detected.

the media, dialysis patients who characteristically have elevated serum lipids are at increased risk as compared to chronically transfused patients.

Blood samples for DEHP analysis were obtained before dialysis and from venous and arterial lines at 1, 2, 3 and 4 hr and immediately after dialysis. Samples of dialysate fluid were obtained from both the inflow and outflow sites during the indicated time intervals. The estimated amount of DEHP received by a patient was calculated from the concentration per milliliter of venous blood times the flow rate multiplied by the duration of dialysis.

As shown in Table 4, DEHP was not demonstrated in the predialysis venous blood samples. All patients showed DEHP in the venous blood but none in the arterial blood during hemodialysis. The estimated total amount of DEHP delivered to each patient during a single dialysis varied from 32 mg to 90 mg. In one year the estimated total amount of DEHP received by a patient would range from 120 mg/kg to 335 mg/kg, a dose 10 to 20 times that which produced liver abnormalities in the rhesus monkey.

Intensive Plasma Exchange

Intensive plasma exchanges were performed with an IBM Blood Cell Separator, 5% albumin being used as replacement fluid. Blood flow rate varied between 50 and 80 cc/min. Blood samples were obtained before plasma exchange and at 30, 60, 90 min and at the conclusion of the exchange.

Three patients requiring maintenance plasma exchange were studied (Table 5). Patient 3 had a total lipid level of 800 mg-% (cholesterol 240 mg-%, phospholipids 225 mg-%, triglycerides 335 mg-%). The estimated total amount delivered to each patient during a single two-plasma volume plasma exchange, varied from 3.3 mg in patients with normal lipid levels to 11.6 mg in the patient with elevated plasma lipids. During a single year this patient receives an estimated 290 mg of DEHP, or 3.3 mg/kg. The patient has undergone plasma exchange for two years.

Discussion

The methods used in previously reported studies call for a more critical assessment of any toxic hazard created by the use of phthalate esters, as it is impossible to establish the significance in man of *in vitro* and *in vivo* studies with an insoluble substance. The amount of DEHP leached into platelets or plasma stored in PVC blood bags is based on the situation that exists in clinical medicine. The rhesus monkey was chosen as an animal model because its red cell survival, platelet preservation characteristics, serum protein pattern and DEHP metabolism are similar to those of the human.

Hepatic disease is often clinically occult due to the remarkable hepatic functional reserve and regenerative capability. The technetium hepatic scintillation scan is a satisfactory method for visually recording abnormal function of diminished perfusion. The latter effect results in impaired hepatic extraction of radioactivity which gives rise to a nonuniform image (*5*). In mild forms of parenchy-

Table 4. DEHP plasma concentration during hemodialysis.

	DEHP plasma concentration, μg/ml					
		Venous DEHP				Estimated total
Patient	Prevenous	1 hr	2 hr	3 hr	3.5 hr	DEHP received, mg
1	ND[a]	3.0	3.8	5.0	4.9	90
2	ND	1.8	4.0	3.5		32
3	ND	2.9	2.5	3.2		57

[a]ND = none detected.

Table 5. DEHP plasma concentration during continuous flow: intensive plasma exchange.[a]

		Plasma DEHP, μg/ml			Estimated total DEHP received, mg	
Patient	Prevenous	30 min	60 min	90 min	Per procedure	Per year
1	ND[b]	2.9	2.6	2.3	3.5	87.5
2	ND	1.7	1.8	2.0	3.3	
3	ND	5.7	4.1	3.2	11.6	290.0

[a]IBM cell separator.
[b]ND = none detected.

mal disease, not manifested by irregular distribution of tracer, changes can be quantitated by determining the relative distribution of radiocolloid between liver and spleen (*9*).

Sulfobromophthalein metabolism and transport may be evaluated in several ways. The standard method determines the retention of BSP at a specific interval, usually 45 min after a single injection (*10*). A more sensitive technique is the utilization of a two-compartmental model for the analysis of BSP kinetics. This was first described in the dog, subsequently validated in the human by Quarfordt et al. and in the rhesus monkey by ourselves. The observation that the normal BSP disappearance curve in the child and immature primate is a single exponential and only changes to a biexponential in the presence of liver disease or with age is of significance to the present study (*8, 11*). At the time this study was completed, none of the rhesus had shed any of their deciduous teeth, nor had they reached sexual maturity. Their average weight was 4.54 kg. One of the monkeys who was part of the study to validate the BSP model had multiple determinations during a three-year period and converted from a single exponential to a biexponential. This occurred at approximately 5.5 years and at a weight of 10.45 kg. The biexponential curves noted in this study and those seen in children with mild liver disease differs from those produced by chronic injury in which the initial slope flattens due to the decrease in the hepatic removal rate (*8*).

Even though the number of animals in each of the groups is too small for valid statistical analysis, a trend is observed in the PVC transfused monkeys. In the present study, prolonged abnormalities in hepatic scan and BSP kinetics occurred following DEHP infusion without changes in standard liver function tests. Six of seven animals had abnormal ^{99m}Tc scan ratios, and six of seven demonstrated abnormal BSP compartmental flow upon completion of transfusion therapy. These findings have persisted for up to 14 months following therapy in three of the four monkeys given PRP and one of the two monkeys transfused with plasma. Two the the three remaining monkeys given PRP continued to have abnormal ^{99m}Tc scan ratios 26 months following the cessation of transfusion therapy. The results of the serial liver biopsies in the PVC transfused monkeys were quite dramatic and all were abnormal except for the initial biopsy on rhesus "G" which was normal. Disturbances of hepatic architecture, the presence of round-cell infiltration, and multinucleated giant cells persisted throughout the follow-up period. The histologic abnormalities have been reported in apparently normal subhuman primates. Although none of the animals in this study had pretreatment biopsies, all seven PVC-transfused rhesus monkeys and none of the six control rhesus had abnormal histology. The probability of this being a chance occurrence is highly unlikely.

Although monkeys transfused with plasma stored in PVC received equivalent doses of DEHP to those in the PVC-PRP group, their duration of exposure was significantly shorter. This may account for the fact that histological changes in liver biopsy were milder and that their liver function as reflected in the kinetics of BSP transport returned to normal during the follow-up period.

The results of DEHP quantitation on the serial liver biopsies of the PVC transfused monkeys shows a decrease in the residual DEHP content after the cessation of transfusion. Although we have not as yet established the metabolic pathway of DEHP in the rhesus monkey, this finding supports the work of Daniel et al. who have demonstrated in the rat and Albro et al. in the African Green monkey that the DEHP is metabolized in part to mono(2-ethylhexyl) phthalate (MEHP) (*12, 13*). This metabolite is a potent inhibitor of liver dehydrogenases, and theoretically may have greater potential for hepatotoxicity than the parent compound.

Based upon a comparison of the abnormalities observed in the platelet-rich plasma and plasma transfused rhesus, a larger dosage over a prolonged time period appears to be more toxic. This is clinically significant to patients undergoing hemodialysis who yearly are exposed to far greater amounts of DEHP than that which produced hepatic toxicity in the rhesus. These patients are more prone to liver disease which cannot be attributed to the hepatitis B surface antigen or non-A, non-B hepatitis.

PVC medical devices have a high degree of reliability and fabrication technology. While the histological and functional abnormalities induced by PVC plasticized with DEHP in the ranges used in this study are mild, they were produced at dose levels observed in transfusion therapy and at lower levels than those that are observed in hemodialysis.

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